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Low Level Wild-Type and Pre-Core Mutant Hepatitis B Viruses and HBeAg Negative Reactivation of Chronic Hepatitis B

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A qualitative and a quantitative mutation-site specific polymerase chain reaction assay (MSSA) was used to detect low level wild-type and pre-core mutant hepatitis B virus (HBV)-DNA. Serum samples from 11 anti-hepatitis B e (anti-HBe)-positive asymptomatic HBV carriers (Group A) and 10 anti-HBe-positive chronic hepatitis B patients who achieved alanine transaminase (ALT) normalization after antiviral therapy (Group B) were tested. Eleven patients had both wild-type and pre-core mutant HBV-DNA (52%, 4 from Group A and 7 from Group B), whereas 3 patients had only pre-core mutant HBV-DNA (14%, 2 from Group A and 1 from Group B) by qualitative MSSA assay. During a 3-year follow-up period, relapses were observed in 3 patients from Group B and intermittent ALT elevation was observed in 4 patients from Group A and 3 patients from Group B. The wild-type HBV-DNA concentration in the patients with reactivation was $10^{2.06\pm2.62}$ copies/ml, whereas that in all patients without reactivation was below 10^2 copies/ml ($P < .05$). The pre-core mutant HBV-DNA concentration in the patients with reactivation was also significantly higher than that in the patients without reactivation ($10^{3.94\pm2.25}$ vs. $10^{0.65\pm1.45}$ copies/ml, $P < .001$). All patients with both HBV-DNA concentrations below 10^2 copies/ml did not exhibit reactivation. Our result suggest that a high prevalence of coexistence of low level wild-type and pre-core mutant HBV-DNA has the potential for reactivation in anti-HBe-positive patients. Furthermore, quantification of wild-type and pre-core mutant HBV-DNA was useful to predict the prognosis of anti-HBe-positive infection and evaluate the efficacy of antiviral therapy. ***J. Med. Virol. 58:332–337, 1999.***

KEY WORDS: mutation-site specific polymerase chain reaction assay (MSSA); HBV-DNA; anti-HBe; antiviral therapy

INTRODUCTION

In patients with chronic hepatitis B, seroconversion from hepatitis B e antigen (HBeAg) to anti-HBe usually indicates a marked decrease of virus replication and activity of liver disease [Hoofnagle et al., 1981]. However, even after this conversion due to interferon (IFN) therapy, relapse of hepatitis occurs occasionally with or without reappearance of HBeAg [Lai et al., 1988; Okamoto et al., 1990b]. Some patients with chronic hepatitis B have persistent viremia and progressive liver disease in the absence of serum HBeAg [Brunetto et al., 1989; Carman et al., 1989]. Furthermore, not only various chemotherapeutic [Galbraith et al., 1975; Wands et al., 1975] or immunosuppressive agents [Nagington et al., 1977; Dusheiko et al., 1983], but also spontaneous reactivation have been well documented in anti-HBe-positive carriers [Davis et al., 1984; Bortolotti et al., 1996]. Recent studies using the polymerase chain reaction (PCR) to amplify very small amounts of viral nucleic acids have shown the persistence of low level of replication with detectable hepatitis B virus (HBV-DNA) in serum and liver, which might explain the reactivation of hepatitis [Kaneko et al., 1989; Gayno et al., 1992; Kuhns et al., 1992; Loriot et al., 1992]. In addition, sequencing HBV-DNA in these HBeAg-negative patients showed pre-core mutations, explaining the lack of HBeAg synthesis but still permitting viral replication [Schlicht et al., 1987; Tong et al., 1991]. The most frequent pre-core mutation is a G-to-A conversion at nucleotide 1896 creating a stop codon (TGG to TAG) in the pre-core region [Carman et al., 1989; Okamoto et al., 1990b]. However, pre-core mutations are not always found in patients with chronic hepatitis B who develop HBeAg-negative reactivation after interferon therapy [Takeda et al., 1990; Santantonio et al., 1991; Talbodec et al., 1995]. Several HBV-DNA quantitative assays such as solution hybridization assay, branched DNA probe assay, and PCR-based

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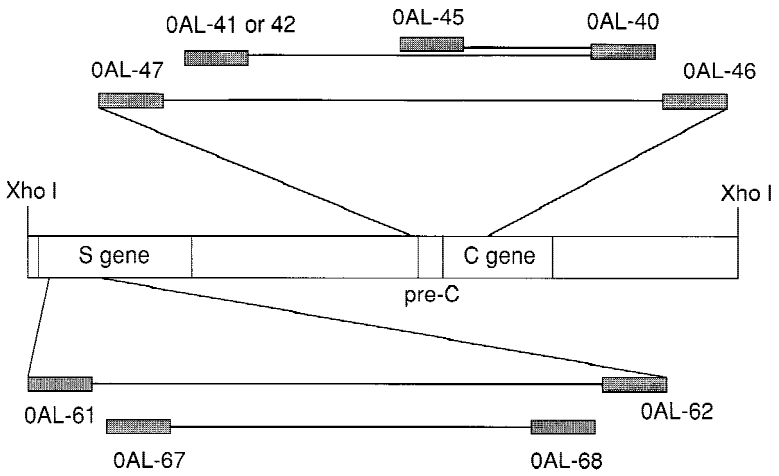

Fig. 1. Location of oligonucleotide primers used in this study. Sequence of oligonucleotide primers are described in the text.

assay have been reported [Kuhns et al., 1988; Urdea et al., 1990; Niitsuma et al., 1997]. However, the relationship between the low level virus concentrations and the potency of the reactivation has not been demonstrated clearly. Furthermore the role of wild-type and pre-core mutant HBV in the reactivation of hepatitis remains unclear. In this study, we used the mutation-site specific PCR assays to qualify and quantify both HBV-DNAs. By using this assay, the relationship between wild-type and pre-core mutant HBV-DNA concentrations and the potency of reactivation was examined in this study.

PATIENTS AND METHODS

Twenty-one patients with chronic type B infection, who were seronegative for HBeAg and seropositive for anti-HBe, were studied. The patients were separated into two groups. Group A (male 5, mean age 50.6 ± 12.5 years): 11 asymptomatic carriers and Group B (male 7, mean age 42.3 ± 11.1 years): 10 chronic hepatitis B patients with therapeutically induced seroconversion were investigated. Asymptomatic carriers were identified as those patients with alanine transaminase (ALT) levels within the normal range (<35 U/L) for 6 months before serum sampling. Nine patients from Group B received alpha interferon therapy and one patient received steroid withdrawal therapy. These patients achieved ALT normalization after therapy. The sera were collected during the ALT normal phase in the two groups of patients and stored at −20°C before qualitative and quantitative PCR analysis. The mean duration between seroconversion and serum sampling from Group B was 35.8 month (7–106 months). All patients were followed as outpatients by our hospital for 3 years after serum sampling. Serum ALT levels and HBV serology were determined sequentially in these patients. HBV serological markers (HBsAg, HBeAg, anti-HBe) were assayed with commercially available enzyme-linked immunosorbent assay kits. Serum HBV-DNA was also analyzed by a nonradioactive solution hybridization technique (Viraprobe kit, TFB).

Qualitative Detection of Wild-Type and Pre-Core Mutant HBV-DNA

The qualitative and quantitative detection of HBV-DNA was carried out in Otsuka Pharmaceutical Diagnostic Research Institute. Serum samples from patients with hepatitis were diluted with an equal volume of 0.2 M NaOH and incubated for 15 min at 37°C. Then the mixture was neutralized with 0.2 M HCl, and a 5.0-μl aliquot of the resulting mixture was subjected to PCR reaction buffer and nested primers within the S region, OAL61 (sense, 5′-CTCGTGTTACAGGCGGGGTTTTTC-3′ nucleotides 65–88), OAL62 (antisense, 5′-GCATAGCAGCAGGATGAAGAGGAAT-3′ nucleotides 274–298), OAL67(sense, 5′-AGAATCCTCACAATACCACAGAGTC-3′ nucleotides 98–122), and OAL68(antisense, 5′-ACGCCGCAGACACATCCAGCGATAAC-3′ nucleotides 240–265). The sequence is numbered from the unique XhoI site in HBV genome (subtype adr) [Kobayashi and Koike, 1984]. OAL61 and OAL62 were used for the first round of PCR and OAL67 and OAL68 for the second round of PCR (Fig. 1). The thermal profile involved 40 cycles of denaturation at 94°C for 1 min, primer annealing at 56°C for 1.5 min, and the primer extension at 72°C for 1.5 min during the first round of PCR, and 40 cycles using the same protocol during the second round. Detection of wild-type and pre-core mutant HBV-DNA was performed by mutation-site specific assay (MSSA) using the principle of mutant-allele-specific amplification [Kinoshita et al., 1994]. PCR was performed with OAL47(sense, 5′-GGCATAAATTGGTCTGTTCA-3′ nucleotides 1660–1679), OAL46(antisense, 5′-ATCAACTCACCCCAACACAG-3′ nucleotides 1951–1971), OAL41(sense, 5′-AAGCTGTGCCTTGGGTGGCTTTA-3′, nucleotides 1748–1770), OAL40(anti-sense, 5′-ATAGCTTGCCTGAGTGCTGT-3′ nucleotides 1931–1950) and OAL45(sense, 5′-ATTCGAGATCTCCTCGACACCG-3′ nucleotides 1853–1874) primers, using a Gene Amp kit (Takara) with a Robocycler PCR machine (Strategene; Nippon Genetics). OAL47 and

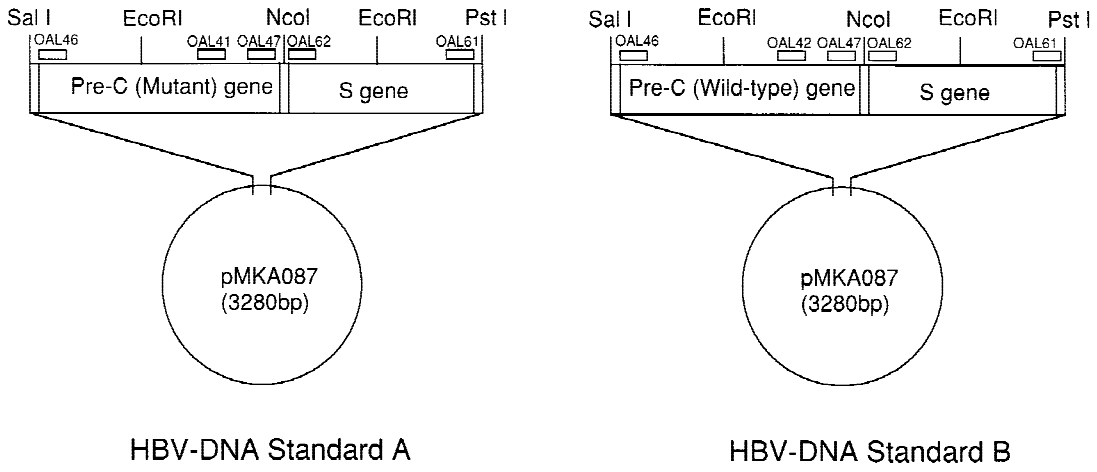

Fig. 2. Construct structure of hepatitis B virus (HBV)-DNA standard A and B and location of oligonucleotide primers used for competitive mutation-site specific assay (CMSSA).

OAL46 were used for the first round of PCR and OAL40, OAL41 and OAL45 for the second round of PCR (Fig. 1). OAL41 encompasses the region between positions 1748 and 1770 in HBV-DNA and is used for trapping the mutation from G to A at the 83rd base in the pre-core region of HBV-DNA. After the nested PCR reaction, 10 μl of the above reaction mixture was electrophoresed on a 3% agarose gel. The identification of a 203 base-pair (bp) band was regarded as demonstrating the presence of HBV with a mutation in the pre-core region of the HBV-DNA genome. In addition, identification of 98 bp was regarded as demonstrating the presence of both pre-core mutant and wild-type HBV genomes in the serum.

Quantitative Detection of Wild-Type and Pre-Core Mutant HBV-DNA

Quantification of total, wild-type, and pre-core mutant HBV-DNA was carried out by means of a competitive MSSA PCR technique (CMSSA). A 5.0-μl aliquot of the HBV-DNA was coamplified with serial dilution of HBV-DNA standard with the PCR protocol described previously. HBV-DNA standard A contains a point mutation from the S region of HBV-DNA (nucleotides 65–298) and a point mutation from the pre-C region of pre-core mutant HBV-DNA (nucleotides 1748–1971). HBV-DNA standard B contains a point mutation from the S region of HBV-DNA and a point mutation from the pre-C region of wild-type HBV-DNA. These point mutations generate a restriction endonuclease site for EcoRI (Fig. 2). After PCR amplification by using pre-C region primers for pre-core mutant HBV-DNA, OAL41, OAL46, and HBV-DNA standard A, sample DNA was digested with EcoRI and the products were electrophoresed on a 3% agarose gel and visualized by ethidium bromide staining. Concentration of pre-core mutant HBV-DNA were determined by the intensity ratio of amplified products from pre-core mutant HBV-DNA (224 bp) and HBV-DNA standard A (133 bp, 91 bp) measured by video-digitizer. Concentrations of wild-type HBV-DNA were determined by the same procedure by using pre-C region primers for wild-type HBV-DNA, OAL42 (sense, 5′-AAGCTGTGCCTTGGGTGGCTTTG-3′, nucleotides 1748–1770) and OAL46, and HBV-DNA standard B. After PCR amplification by using S region primers and either HBV-DNA standard A or B, concentrations of total HBV-DNA were determined by the intensity ratio of amplified products from HBV-DNA (234 bp) and HBV-DNA standard (117 bp).

RESULTS

Reactivation of hepatitis were observed in four patients from Group A and six patients from Group B during the follow-up period (Table I). Among these patients, all four patients from Group A and three patients from Group B showed intermittent ALT elevation. The other three patients from Group B showed sustained ALT abnormalities after onset of reactivation, that is, relapse. Two of these patients (patient 1 and patient 3 from Group B in Table I) became seropositive for HBeAg and seronegative for anti-HBe after reactivation.

Qualitative Detection of Wild-Type and Pre-Core Mutant HBV-DNA

Nested PCR analysis with the use of S region primers detected HBV-DNA in 14 (67%) of the 21 patients. In the same sample, HBV-DNA was detected in 7 (33%) of the 21 patients by solution hybridization method. When the MSSA assay was used, both wild-type and pre-core mutant HBV-DNA were positive in 11 (52%) of 21 patients and only pre-core mutant HBV-DNA was positive in the other 3 patients. Eight of 11 both wild-type and pre-core mutant HBV-DNA positive patients (73%, 3 from Group A and 5 from Group B) and 2 of 3 pre-core mutant HBV-DNA positive patients (67%, 1 from Group A and 1 from Group B) showed reactivation. In contrast both HBV-DNA negative patients maintained normal serum ALT concentrations during the follow-up period.

TABLE I. Qualitative and Quantitative Detection of Total, Wild-Type, and Pre-Core Mutant HBV-DNA in Patients With anti-HBe-Positive Asymptomatic Carriers (Group A) and Anti-HBe-Positive Chronic Hepatitis B Patients With Response After Antiviral Therapy (Group B)

Patient	Age (years)/Gender	Total HBV-DNA Quality/Quantity (×100 copies/ml)	Wild-type HBV-DNA Quality/Quantity (×100 copies/ml)	Pre-core mutant HBV-DNA Quality/Quantity (×100 copies/ml)	ALT prognosis
Group A					
1	66/F	++/40,000	–/<1	++/40,000	Intermittent elevation
2	41/M	+/40	+/<1	+/20	Intermittent elevation
3	49/M	+/10	+/10	+/10	Intermittent elevation
4	56/M	+/10	+/<1	+/<1	Intermittent elevation
5	40/M	+/30	+/<1	+/30	No change
6	29/F	+/30	–/<1	+/<1	No change
7	69/M	–/<1	–/<1	–/<1	No change
8	52/F	–/<1	–/<1	–/<1	No change
9	47/F	–/<1	–/<1	–/<1	No change
10	43/F	–/<1	–/<1	–/<1	No change
11	65/F	–/<1	–/<1	–/<1	No change
Group B					
1	47/M	++/2,000,000	++/1,000,000	++/600,000	Relapse
2	36/M	+/50	+/40	+/6	Relapse
3	38/M	+/40	+/<1	+/40	Relapse
4	51/M	++/1,000	–/<1	++/1,000	Intermittent elevation
5	66/M	+/40	+/10	+/20	Intermittent elevation
6	48/F	+/30	+/10	+/10	Intermittent elevation
7	32/F	+/100	+/<1	+/50	No change
8	42/F	+/10	+/<1	+/<1	No change
9	34/M	–/<1	–/<1	–/<1	No change
10	29/M	–/<1	–/<1	–/<1	No change

HBV, hepatitis B virus; HBe, hepatitis B e; ALT, alanine transaminase; F, female; M, male.

Quantitative Detection of Wild-Type and Pre-Core Mutant HBV-DNA

HBV-DNA concentrations were less than the detection limit in 6 (55%) of the 11 wild-type HBV-DNA-positive patients and in 3 (21%) of the 14 pre-core mutant HBV-DNA-positive patients using CMSSA assay. The mean total HBV-DNA concentration was $10^{2.80\pm2.40}$ copies/ml (Group A: $10^{2.11\pm2.24}$, Group B: $10^{3.57\pm2.46}$ copies/ml). The mean wild-type and pre-core mutant HBV-DNA concentrations were $10^{0.98\pm2.05}$ copies/ml (Group A: $10^{0.27\pm0.91}$, Group B: $10^{1.76\pm2.67}$ copies/ml) and $10^{2.22\pm2.48}$ copies/ml (Group A: $10^{1.49\pm2.26}$, Group B: $10^{3.02\pm2.58}$ copies/ml), respectively. The relationship between the total, wild-type, and pre-core mutant HBV-DNA concentrations and reactivation is shown in Figure 3. The total HBV-DNA concentration in the patients with reactivation was significantly higher than that in the patients without reactivation ($10^{4.49\pm1.82}$ vs. $10^{1.27\pm1.77}$ copies/ml, $P < .001$). The wild-type HBV-DNA concentration in the patients with reactivation was $10^{2.06\pm2.62}$ copies/ml, whereas the concentration in the patients without reactivation was below 10^2 copies/ml ($P < .05$). The pre-core mutant HBV-DNA concentration in the patients with reactivation was also significantly higher than that in the patients without reactivation ($10^{3.94\pm2.25}$ vs. $10^{0.65\pm1.45}$ copies/ml, $P < .001$). All patients with total HBV-DNA concentrations below 10^2 copies/ml maintained normal serum ALT concentrations. In contrast, all patients who had more than 10^5 copies/ml either total, wild-type, or pre-core mutant HBV-DNA levels showed reactivation.

DISCUSSION

In patients with chronic HBV infection, seroconversion from HBeAg to anti-HBe is usually associated with the decrease of viremia and quiescence of hepatitis. Serum HBV-DNA concentration in the anti-HBe-positive stage is significantly lower than that in the HBeAg-positive stage. Therefore a highly sensitive assay is necessary to detect low level HBV-DNA in the anti-HBe-positive stage. In this study, 67% of patients (55% of anti-HBe-positive asymptomatic HBV carriers and 80% of anti-HBe-positive chronic hepatitis B patients), had detectable HBV-DNA as detected by nested PCR assay. In contrast, HBV-DNA were detected in 33% of patients by solution hybridization assay. Eleven (79%) of 14 HBV-DNA-positive patients had both wild-type and pre-core mutant HBV-DNA by MSSA assay. Wild-type HBV-DNA was detected even in the patients with anti-HBe-positive asymptomatic carriers (those who had natural seroconversion). When CMSSA assay was used, wild-type HBV-DNA concentrations were less than the detection limit in 55% of wild-type HBV-DNA-positive patients by MSSA assay. Our data indicate that a minute amount of wild-type HBV still coexists with pre-core mutant HBV even in anti-HBe-positive patients. The mechanisms controlling the appearance of the pre-core mutation are not well understood, but these pre-core mutations seem to be more favored under immune pressure. In particular, interferon antiviral therapy might favor the emergence of an HBV mutant by selection pressure, giving a selected advantage to escape the immune response [Takeda et

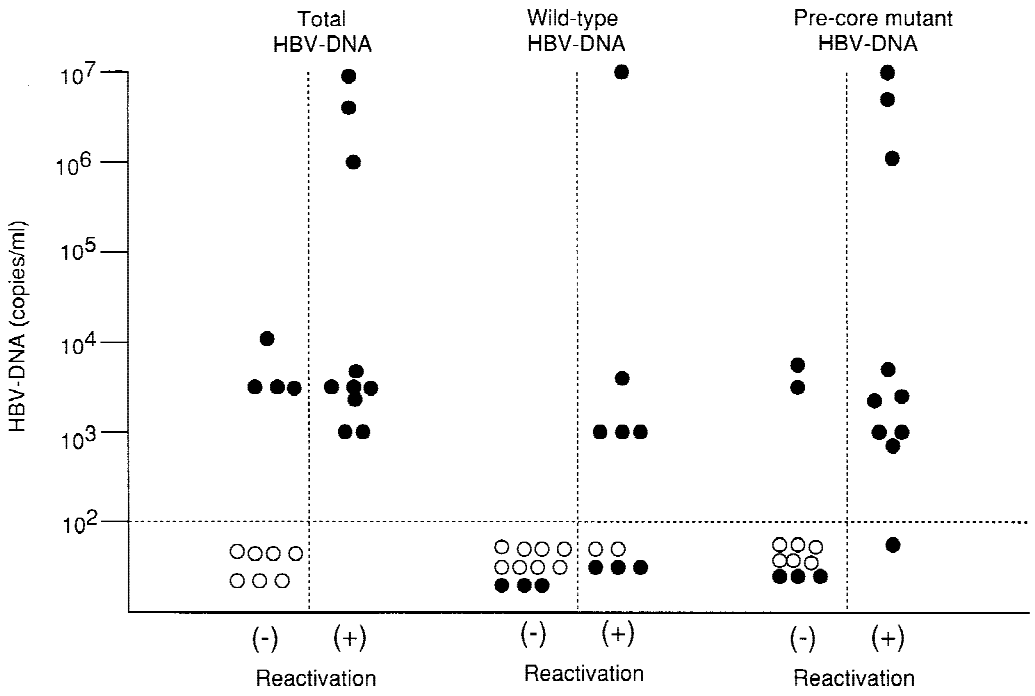

Fig. 3. The total, wild-type and pre-core mutant hepatitis B virus (HBV)-DNA concentrations by competitive mutation-site specific assay (CMSSA) in the patients with or without reactivation. ●, HBV-DNA positive by qualitative mutation-site specific assay (MSSA); ○, HBV-DNA negative by qualitative MSSA.

al., 1990; Santantonio et al., 1991; Tran et al., 1991]. Our results suggest that coexistence of wild-type and pre-core mutant HBV is advantageous to avoid immune pressure.

The relationship between the virus concentrations and the potential for reactivation is clinically important. In this study, significant correlation was observed between total, wild-type, and pre-core mutant HBV-DNA levels and reactivation. Patients infected with either total, wild-type, or pre-core mutant HBV-DNA concentrations of more than 10^5 copies/ml had high potential for reactivation, whereas patients with those below 10^2 copies/ml had low potential for reactivation. As shown in the result, all five patients with successful quantification of wild-type HBV-DNA showed reactivation. Among them, two patients showed reactivation similar to the pattern of relapse. In addition, even a patient with a minute amount of wild-type HBV-DNA, below the detection limit of CMSSA assay, had relapse accompanied by the reappearance of HBeAg. This finding suggests that the presence of qualitative wild-type HBV-DNA still has potency of reactivation in a pattern similar to relapse. Talbodec et al. [1995] reported that a pre-core mutant was not always detected in patients with HBeAg-negative reactivation. Our result is consistent with their findings. The role of pre-core mutant HBV in the severity of liver disease remains unclear [Brunetto et al., 1991; Naoumov et al., 1992]. In this study, patients infected with quantitative pre-core mutant HBV showed reactivation resembling the pattern of intermittent ALT elevation. These findings suggest that the proportion of pre-core mutant to total HBV may correlate with the pattern of reactivation. It was notable that patients infected with only pre-core mutant HBV had relatively high levels of pre-core mutant HBV-DNA (patient 1 from Group A and patient 4 from Group B in Table I). The concentrations of pre-core mutant HBV-DNA in these patients were relatively high as detectable by solution hybridization assay (data not shown). Further study is necessary to examine the presence of mutation in the core promoter, which is characterized as a HBeAg-negative phenotype with a high viral load, as described by Okamoto et al. [1990a]. Another possibility is that elimination of wild-type HBV may enable pre-core mutant HBV to proliferate efficiently.

The development of cirrhosis and hepatocellular carcinoma is reported in anti-HBe-positive patients despite sustained ALT normalization and HBsAg clearance [Adachi et al., 1992]. Furthermore, reactivation is observed in anti-HBeAb-positive asymptomatic carriers after immunosuppressive or cytotoxic treatment. Quantitative detection of wild-type and pre-core mutant HBV-DNA may be useful to find such high-risk patients. In addition, this assay may be useful to evaluate the efficacy of antiviral therapy and to predict the prognosis. Monitoring of HBV-DNA level may be more useful, because fluctuation of viral load is suspected in some cases and occurrence of hepatitis is affected by the host immune response [Bertoletti et al., 1994; Mels et al., 1994].

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